Role of X-linked inhibitor of apoptosis (XIAP) in frozen and thawed dormant and normal-hatched murine blastocysts

Role of X-linked inhibitor of apoptosis (*XIAP*) in frozen and thawed dormant and normal-hatched murine blastocysts

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ABSTRACT

Cryo-injury of mammalian blastocysts occurs during cryopreservation and induces apoptosis in trophoblast cells. This damage affects subsequent embryo development or may even cause death before implantation. X-linked inhibitor of apoptosis (*XIAP*) is an anti-apoptosis gene that has been widely studied in cancer research. However, only a few studies have investigated the activity of XIAP in cryopreservation. In this study, we investigate the role of *XIAP* in frozen and thawed murine blastocysts. A total of 1630 blastocysts were divided into fresh and freeze-thaw groups, and *XIAP* expression was investigated using qPCR, Western blot and confocal analyses. In addition, the effect of the embelin (a XIAP inhibitor) was also evaluated by co-culturing 390 dormant blastocysts. XIAP protein is primarily localized to the mitochondria of trophoblastic cells. Gene and protein expression is significantly down-regulated in blastocysts after cryopreservation, whereas embelin has negative effect on their survivals. These findings further broaden the understanding of mammalian embryonic cryopreservation.

Keywords: Mouse; cryopreservation; dormant embryos; XIAP; apoptosis

1. Introduction

In recent years, cryopreservation of mammalian oocytes and embryos has become a routine technology for assisted-reproduction in both animals and humans. However, injury to embryos can occur during the freeze-thaw stages of this process, resulting in destruction of the cellular cytoskeleton and a reduced embryonic survival rate [15]. Previous studies have compared fresh with cryopreserved and thawed blastocysts [16, 21], and the ability of re-expansion in blastocysts after thawing has been evaluated as a prerequisite to survival [9]. Indeed, apoptosis can be assessed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). However, blastocysts consist of hundreds of cells and the TUNEL data might give unreliable estimates because averaged over all blastomeres [14].

X-linked inhibitor of apoptosis (*XIAP*) is as a potent and versatile inhibitor of apoptosis proteins (IAPs) [12]. Embryonic diapause has been used in mice as model to investigate *XIAP* expression in normal and dormant embryos following cryopreservation and a down-regulation has been found in the latter [27]. Cryopreservation induces DNA fragmentation in surviving blastocysts by re-expansion, and it is also associated with membrane damage that leads to cell loss. Moreover, there is an increased incidence of apoptotic events in the trophectoderm (TE) cells of cryopreserved blastocysts [9].

Despite this preliminary study, little information is known on the role of XIAP expression in cryopreserved embryos. For instance, some studies suggested that XIAP plays an important role for apoptotic regulatory molecules, regulating trophoblast survival and influencing embryonic development [3]; [6]; [20]; [23]. Silasi et al. assessed XIAP expression in fresh-frozen ovarian cancer samples using laser-capture microdissection [19], while Arroyo et al.

suggested that decreased XIAP activation could be associated with increased placental apoptosis [1].

Embelin is a polyphenolic compound that inhibits XIAP by binding the Smac site in the BIR3 domain of XIAP molecules [7]; [18]. Previous studies have demonstrated that embelin initiates anti-inflammatory and anti-oxidative effects [10]. It also has an extensive anti-tumor effect inducing caspase 3 and 9 activation and decreasing XIAP expression in killing prostate tumor cells [2].

Collectively, these reports suggested that further investigation of the *XIAP* gene could provide novel insights into cryopreservation in mice blastocysts. Therefore, in the present study, both molecular and cellular approaches were applied to acquire a more detailed understanding of the role of XIAP in cryopreservation.

2. Materials and methods

2.1. Ethics approval

Animal experiments were performed at the Animal Care Facility of Beijing University of Agriculture. Housing and treatments of the mice were in accordance with Beijing Laboratory Animal Management Committee guidelines for the care and use of laboratory animals.

2.2. Animal models and blastocysts

A total of 150 female mice (ICR; Beijing Vital River Laboratory Animal Technology Co., Ltd.; 6 weeks) were housed in the Institutional Animal Care Facility of Beijing University of Agriculture (SYXK Beijing 2010- 0003). Food and water were available. The schematic diagram of the experimental design is reported in figure 1. 10 IU of pregnant mare's serum

gonadotropin (PMSG; Ningbo Sansheng Pharmaceutical Co., Ltd； China) was used to treat superovulation by intraperitoneal injection on day 1 and human chorionic gonadotropin (HCG; Ningbo Sansheng Pharmaceutical Co., Ltd; China) was given on day 3 [5], followed by the mating with males. Successful mating had presence of a copulation plug on the morning of day 4. Then 10 IU of anti-pregnant mare's serum gonadotropin (A-PMSG; Tianjin Laboratory Animal Center; China) on 09.00 h was injected to reduce the negative impact of residual estrogen during superovulation [13]; [11]. On the pregnancy of day 4 (08.00–09.00 h), in total 100 females were ovariectomized and subsequently injected daily until day 7 by subcutaneous progesterone (2mg per mouse per day; P_4) in sesame oil for collecting dormant blastocysts. Approximately 30 females were left intact to collect normal blastocysts [5]. A total of 1010 dormant blastocysts were collected on day 8, as well as 620 normal blastocysts were directly collected on day 5.

2.3. Freezing and thawing of embryos

The freezing step with cryo-protectant (ECEG-100; ICPBio; New Zealand) was performed on 310 normal and 310 dormant blastocysts, as reported by Gu et al. [5]. Before performing the experiments, straws containing normal and dormant blastocysts respectively were removed from liquid nitrogen, gently shaken for 5–10 s and moved to a 35 °C water bath for 10 s for thawing [5]. Afterwards, the blastocysts were quickly transferred to a glass dish. Both the cryo-normal and cryo-dormant blastocysts were cultured *in vitro* for 2 hours to re-expand from the cryopreservation. Then, both re-expanded and non-re-expanded blastocysts underwent q-PCR and Western blot analysis.

2.4. Quantitative real-time PCR analysis (qPCR)

XIAP gene expression was analyzed from four groups of embryos: fresh normal-hatched and dormant embryos; cryo-normal and cryo-dormant embryos. Fresh embryos were immediately used for RNA isolation, whereas the cryopreserved embryos were washed from the cryoprotectants (CPA) and had time to re-expand during 2h of culture, before Real-time PCR. q-PCR was performed on 100 embryos per group (without distinguishing re-expanded and not re-expanded embryos) [5] by using the following *XIAP* gene-specific primers: forward, 5′- TCC CAT GTG CTA CAC CGT CA -3′; and reverse, 5′- GCA GAT TAC TTA AAG TTC GCT CCC -3′ (GenBank accession number NM_001301641). The housekeeping gene was *GAPDH* (GenBank accession number NM_008084), and the primers were forward, 5′- TGG CAA AGT GGA GAT TGT TGC C -3′; and reverse, 5′- AAG ATG GTA ATA AAC TTC CCG -3′. q-PCR was performed in triplicate (technical replicate) to obtain estimates of variation (SEM). Results were indicated as fold-change relative to the mean according to Gu et al. [5].

2.5. Western blot analysis of blastocysts

The cryopreserved embryos were thawed from the cryoprotectants (CPA) and had time to re-expand during 2 hours of culture, before Western blot analysis. A total of 200 blastocysts from each group (without distinguishing re-expanded and not re-expanded embryos) were used to isolate total proteins and run in triplicate on 10% SDS-PAGE (technical replicate), to obtain estimates of variation (SEM). Bands were transferred to a PVDF membrane (Millipore, MA, USA) and treated with primary and secondary antibodies as reported by Gu et al. [5]. Band

intensity values were analyzed by using Image J software (National Institute of Health; Bethesda, MD, USA).

2.6. Evaluation of the effect of embelin on embryonic viability after cryopreservation

First a pilot trial was performed to test the effective concentration of embelin. Embelin (Sigma-Aldrich) was added to Whitten's solution to reach final concentrations of 0, 20, 50, 100, and 200 μM, respectively, and approximately 30 dormant embryos per concentration were incubated during 4 h embryo culture. We looked at post-thaw embryos survival (embryonic viability after cryopreservation) to evaluate the effect of embelin. 50μM appeared to be the 'optimal' concentration due to the decreasing post-thaw embryos survival following co-cultured in vitro for 4 hours.

Based on the pilot experiment, 50μm was chosen in the experiment to study the effect of XIAP inhibition by embelin on post-thaw embryo survival. As embelin was added to the culture medium from a stock solution of embelin dissolved in DMSO, 50 μM of embelin led also to the presence of 2% DMSO. The effect of the DMSO vehicle was tested in a separate group. Thus, post-thaw embryo survival was estimated in four groups, containing 100, 89, 100, 101 dormant embryos: 1a) Embryos frozen without treatment (control); 1b) same as 1a, but embryos were firstly cultured in-vitro for 4 h; 2) embryos were incubated with 50μM embelin during 4 h prior to freezing and thawing; 3) embryos were incubated with 2% DMSO vehicle during 4 h prior to freezing and thawing, as shown in Fig. 1. The re-expansions (live post-thaw blastocysts) were observed by microscope and the survival rates were calculated for three subgroups of embryos per group (each subgroup having roughly one-third of the number of

embryos per group) in order to obtain an estimate of variation and present the survival rates per group as means ± SEM.

2.7. Immunofluorescence staining and confocal microscopy

The distribution of XIAP was observed by performing immunofluorescence staining of blastocysts using an anti-XIAP antibody (ab2541, Abcam) [25]. Slides were scanned using a laser microscope (Zeiss LSM 710; Jena, Germany), and images were analyzed with Zeiss LSM image browsing software.

2.8. Statistical analysis

Data are presented as mean ± SEM. Analysis of variance (ANOVA) was used to establish differences among groups of analyzed blastocysts for all the performed tests (q-PCR and Western blot), followed by a Student-Newman-Keuls test using SPSS software. A *p*-value of less than 0.05 was considered to be statistically significant.

3. Results

1. Expression of XIAP Is Decreased After Cryopreservation.

It is well-known that *XIAP* is a key gene that regulates cell apoptosis, and that this role may affect the cryopreservation survival rate. To investigate the relationship between *XIAP* and cryopreservation in mice blastocysts, qPCR, confocal and Western blotting were performed. In addition, the XIAP inhibitor, embelin, was co-cultured with blastocysts to evaluate its effect on the survival rate.

As shown in Fig. 2A, *XIAP* expression was down-regulated in the cryo-normal and cryo-dormant groups in comparison with the normal-hatched and dormant controls ($p < 0.01$). *XIAP* expression in cryo-dormant blastocysts groups was also significantly lower than in the normal-hatched groups. Western blot results (Fig. 2, B and C) confirmed this pattern, with *XIAP* down-regulated in cryo-normal ($p<0.05$) compared with non-frozen-normal embryos. Also, the expression in the cryo-dormant group appeared to be lower than in the non-frozen-dormant group, but this difference was not significant. Expression in the cryo-dormant group was significantly lower compared with the non-frozen normal group ($p<0.05$).

2. XIAP Inhibitor (Embelin) Is Negative Effect on Survival.

XIAP expression in western blots appeared to be less reduced in frozen-dormant embryos than in frozen-normal embryos, as the difference between frozen and fresh was not significant in dormant embryos (whereas it was significant in normal embryos) and frozen-dormant embryos tended ($p=0.07$) to have a higher expression compared with frozen-normal embryos. As XIAP is an inhibitor of apoptosis, its expression may be related to the level of post-thaw embryo death. Therefore, embelin addition co-cultured with dormant embryos *in-vitro* prior to freezing was used to evaluate its effects on survival rates after thawing and to explore the role between XIAP and cryopreservation. As shown in table 1, there was no effect on the survival rate when blastocysts were co-cultured *in vitro* in the 2% DMSO control ($p>0.05$) prior to freezing, whereas addition of embelin prior to freezing decreased the survival rate of blastocysts ($p<0.05$). No significant differences were observed between the two control groups

for frozen/thawed without any pre-treatment (Control 0 h) and with Whitten's solution culture *in-vitro* for 4 h prior to frozen/thawed (Control 4 h).

3. XIAP Localizes to Multiple Small Foci Inside TE Cells, But under the Submembrane of Cytosol.

Confocal microscopy was used to determine the localization of XIAP in blastocysts from each group. XIAP was present only in TE cells (Fig. 3), in multiple foci in the cytoplasm of trophoblast cells. More specifically, the XIAP protein was localized to mitochondrial foci in the periphery of the TE cells. In addition, XIAP expression in cryo-normal and cryo-dormant embryos were visible and stronger than in groups prior to freezing.

4. Discussion

It was previously reported that cryopreservation down-regulates the transcription of most genes, but up-regulates expression of heat shock proteins. This effect was caused by freezing and thawing rather than by exposure to cryo-protectants [17]; [26]. The current study shows that *XIAP* expression was down-regulated in both cryo-normal and cryo-dormant murine blastocysts, as detected by both qPCR and Western blot analysis.

In our Western blot analysis, XIAP expression appeared to be higher in the cryo-dormant group than in cryo-normal blastocysts; the estimated difference showed a tendency to be significant ($p=0.07$). In addition, no significant difference of XIAP expression was detected between the frozen and fresh dormant embryos (whereas it was significant in normal embryos). Cryopreservation resulted in a decrease in XIAP expression in normal embryos but not in

dormant embryos, this may explain the higher survival of dormant embryos after cryopreservation. Therefore, we proposed that *XIAP* gene expression could be one reason which affects the cryopreservation and the related survival rate. In addition, embelin significantly reduced tolerance to freezing in mouse blastocysts, while no significant reduction was detected in the 2% DMSO control group. Hussain et al. suggested that embelin has an anti-tumor effect by inducing apoptosis of cancer cells and inhibiting their proliferation [8]. However, it was also found that embelin treatment was associated with decreased XIAP expression, even though it did not induce or enhance apoptosis. We found that incubation of embryos with embelin prior to freezing reduced the survival rate, suggesting that specific XIAP inhibition by embelin increased apoptosis and reduced embryonic survival after cryopreservation.

Confocal microscopy revealed that XIAP localizes to multiple small foci inside TE cells, and that these foci are in mitochondria within the TE cells of murine blastocysts. These results are consistent with those of Van Blerkom [22]. In light of their diverse activities in normal cells, it appears that mitochondria may be central determinants of embryonic development. In mammalian cells, mitochondria-associated IAP antagonists play crucial roles in these processes. Furthermore, upon binding, an unusual septin-like mitochondrial proteins promote the ubiquitin-mediated degradation of XIAP; as exemplified by the *Drosophila* IAP antagonists [4]. ARTS (a member of the septin family of proteins) knockout mice have elevated XIAP levels, resulting in enhanced resistance to cell death [24]. These results suggested that *XIAP* gene has potential link with mitochondrial regulation and cell death. Indeed, our study was provided the similarly potential link relationship between *XIAP* gene and cell death. However,

the exact mechanism which affects embryonic apoptosis and cell death requires further clarification.

In conclusion, the relationship between expression of XIAP in murine blastocysts and cryopreservation has been further investigated. It was demonstrated that cryopreservation reduces the expression of XIAP and decreases the survival rate of blastocysts. Furthermore, XIAP inhibiter observed negative effect on embryonic survival during cryopreservation. Interestingly, XIAP was localized to the mitochondria of trophoblast cells. This study places the normal hatched and dormant embryos as target for exploring the relationship between *XIAP* gene and cryopreservation.

Acknowledgments

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Conflicts of Interest

There are no conflicts of interest.

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Figure Legends

Fig. 1. Schematic diagram manifesting experimental design flow. One hundred fifty mice were super-ovulated by in intraperitoneal injection with PMSA (14.00-15.00 h). Mice were injected with hCG and mated after 48 h. On the morning of day 4 (9.00 h), checking plug and intraperitoneal injection with A-PMSG. One hundred mice were ovariectomized and subsequently daily injected with 2mg per mouse of subcutaneous progesterone (P_4). Dormant blastocysts were collected by flushing each uterine cornua on the morning of day 8. Conversely, normal hatched blastocysts were collected from 30 mice by flushing the uteri on the morning of day 5 (9.00 h).

Four experimental flows were set up (1. Realtime PCR, 2. Western Blot; 3. Dormant Blastocysts Culture in vitro; 4. Immunofluorescence and Confocal) and numbers of blastocysts were showed in brackets.

Fig. 2. Analyses of *XIAP* gene expression and Western blot in dormant and normal-hatched embryos. A) *XIAP* transcription was analyzed in various blastocyst groups. Concentrations of *XIAP* mRNA were normalized to those of *GAPDH*. B and C) Western blot profiles of XIAP (cropped blots) normalized to β-actin. Values are expressed as mean ± SEM, * $p < 0.05$, ** $p < 0.01$.

Fig. 3. Localization of XIAP in mouse embryos (10× and 20× magnification). Images show XIAP antigen labeled in green, propidium-iodide-labeled nuclei in red, and merging of the images.

325

Table 1. Survival rates of dormant embryos were evaluated in co-culture with embelin addition used prior to freezing.

	Different Treatments			
	Control (0 h)	Control (4 h)	Embelin (4 h)	2% DMSO
No. of blastocysts	100	89	100	101
Surviving rates of post-thawing (%)	70.00±3.50[a]	65.33±0.82[a]	45.67±4.37[b]	65.00±2.52[a]

Note: Data are presented as means ± SEM. Different letters identify statistically significant values. The four groups are all dormant embryos as follows: 1a) Frozen/thawed without any pre-treatment (0h control); 1b) culture in-vitro for 4 h prior to frozen/thawed; 2) Embelin addition in co-culture for 4 h used prior to freezing/thawing; 3) 2% DMSO (final concentration) addition in co-culture for 4 h used prior to freezing/thawing.

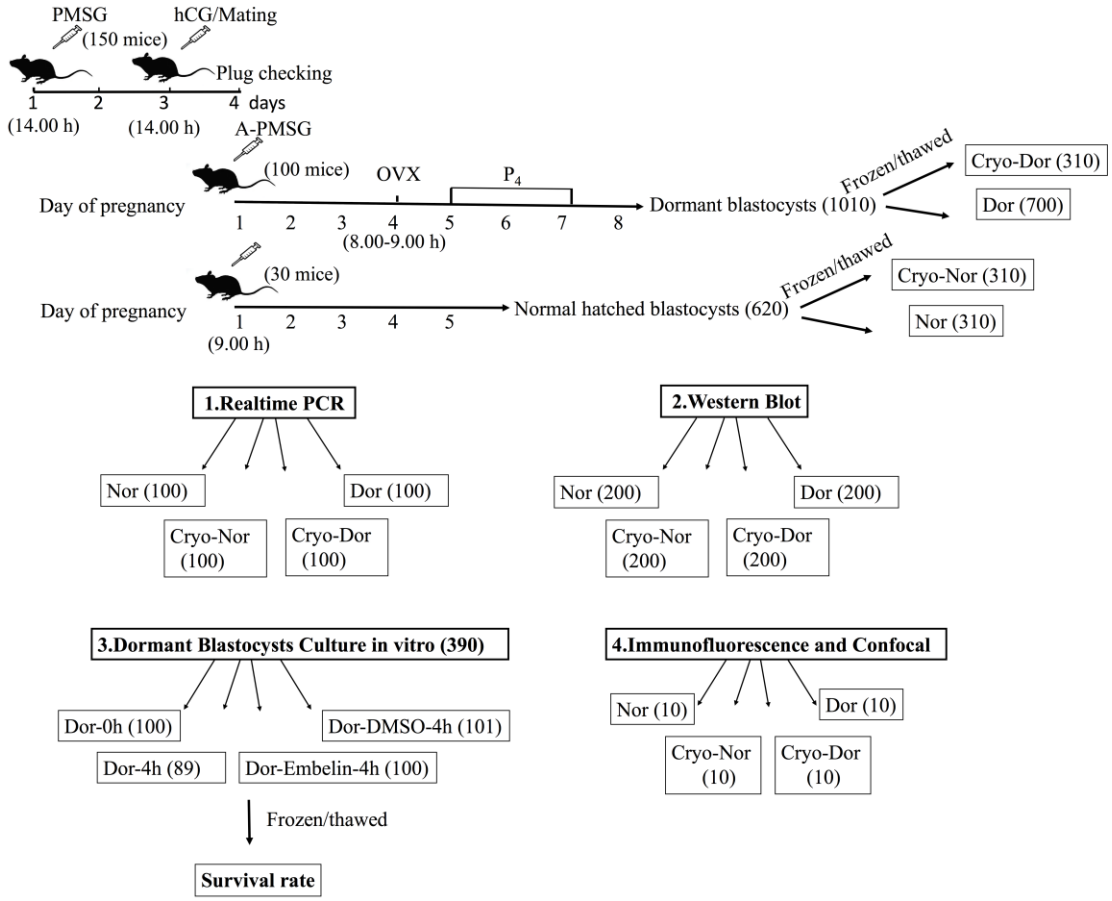

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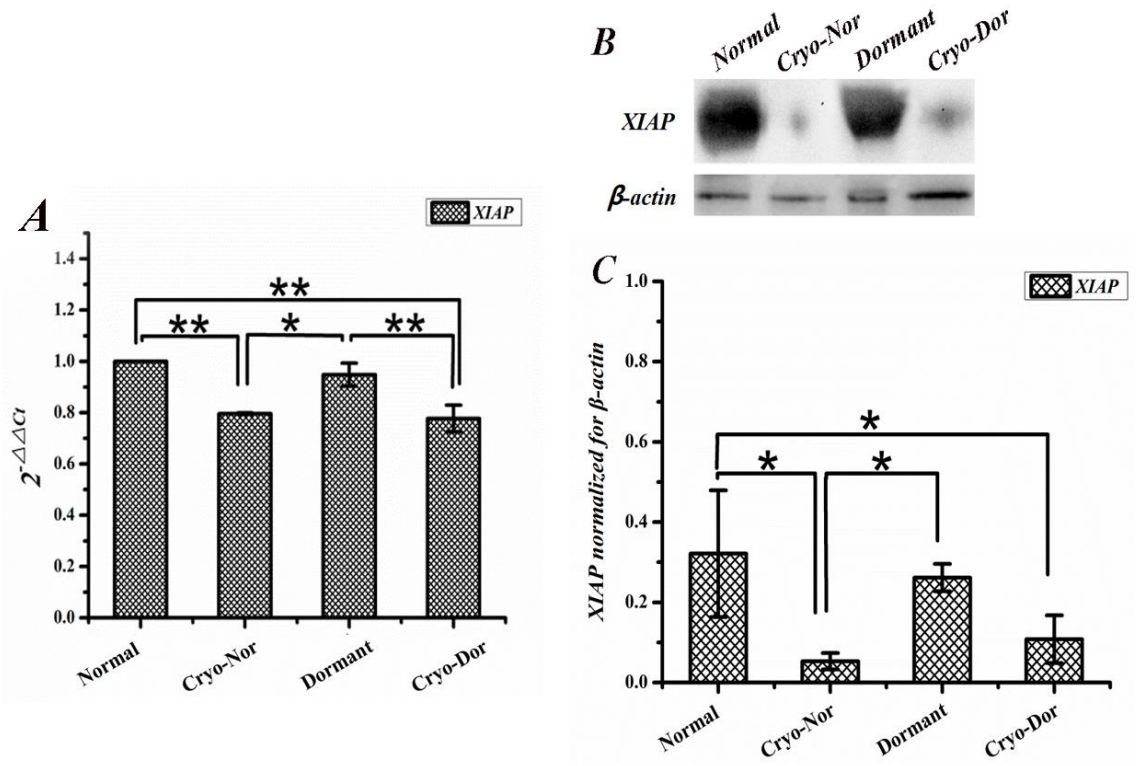
Figure 1

337


A
XIAP
1.4
1.2
1.0
0.8
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2^-ΔΔCt
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Normal
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B
Normal
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Figure 2

338

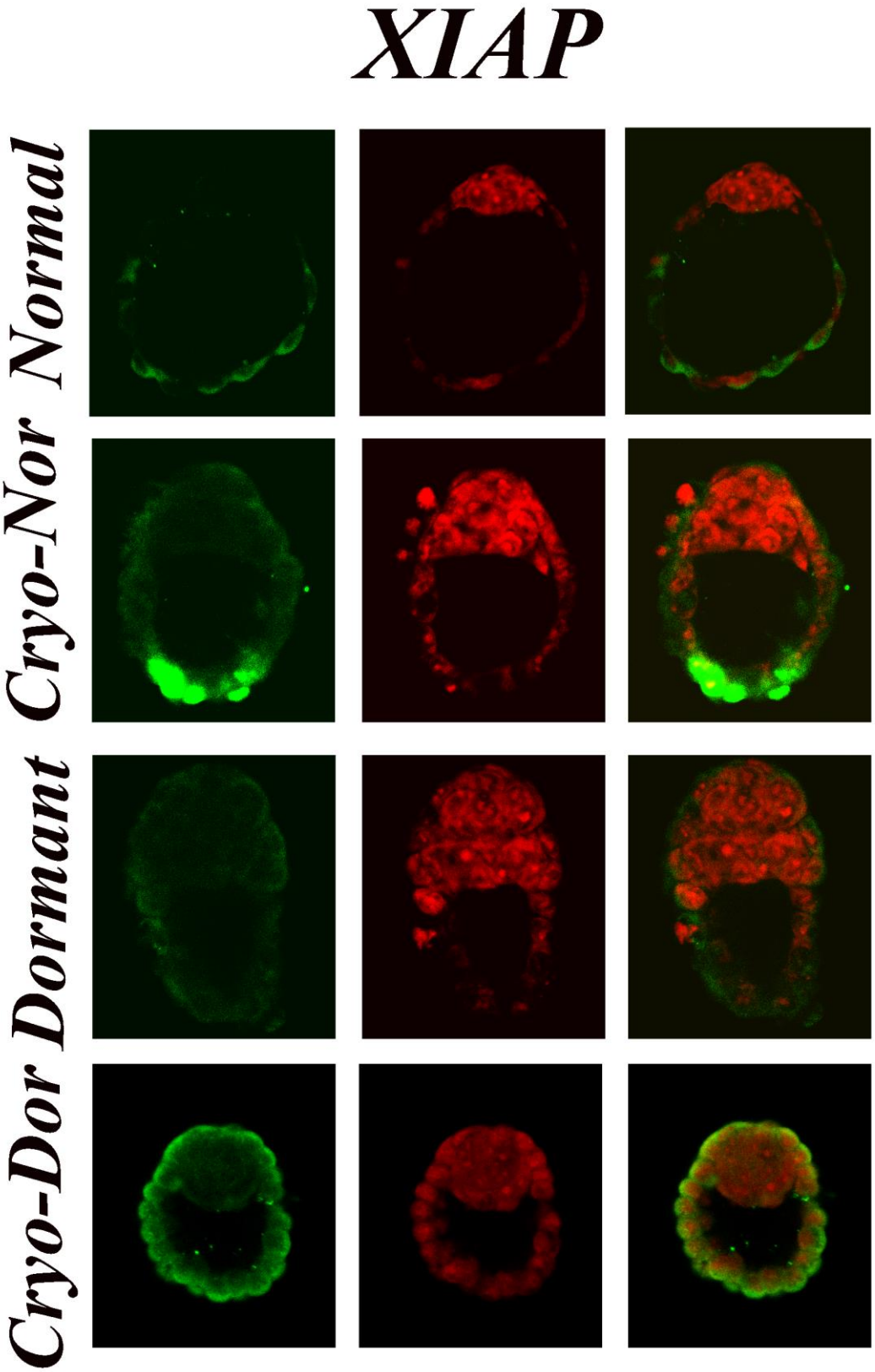

Figure 3